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ABSTRACT

Background: The pathophysiology of necrotizing enterocolitis (NEC) remains poorly understood.

Objective: We assessed the relation between feeding strategies, intestinal microbiota composition, and the development of NEC.

Design: We performed a prospective nationwide population-based study, EPIPAGE 2 (Etude Epidémiologique sur les Petits Ages Gestationnels), including preterm infants born at <32 wk of gestation in France in 2011. From individual characteristics observed during the first week of life, we calculated a propensity score for the risk of NEC (Bell's stage 2 or 3) after day 7 of life. We analyzed the relation between neonatal intensive care unit (NICU) strategies concerning the rate of progression of enteral feeding, the direct-breastfeeding policy, and the onset of NEC using general linear mixed models to account for clustering by the NICU. An ancillary propensity-matched case-control study, EPIFLORE (Etude Epidémiologique de la flore), in 20 of the 64 NICUs, analyzed the intestinal microbiota by culture and 16S ribosomal RNA gene sequencing.

Results: Among the 3161 enrolled preterm infants, 106 (3.4%; 95% CI: 2.8%, 4.0%) developed NEC. Individual characteristics were significantly associated with NEC. Slower and intermediate rates of progression of enteral feeding strategies were associated with a higher risk of NEC, with an adjusted OR of 2.3 (95% CI: 1.2, 4.5; $P = 0.01$) and 2.0 (95% CI: 1.1, 3.5; $P = 0.02$), respectively. Less favorable and intermediate direct-breastfeeding policies were associated with higher NEC risk as well, with an adjusted OR of 2.5 (95% CI: 1.1, 5.8; $P = 0.03$) and 2.3 (95% CI: 1.1, 4.8; $P = 0.02$), respectively. Microbiota analysis performed in 16 cases and 78 controls showed an association between *Clostridium neonatale* and *Staphylococcus aureus* with NEC ($P = 0.001$ and $P = 0.002$).

Conclusions: A slow rate of progression of enteral feeding and a less favorable direct-breastfeeding policy are associated with an increased risk of developing NEC. For a given level of risk assessed by propensity score, colonization by *C. neonatale* and/or *S. aureus* is significantly associated with NEC. This trial (EPIFLORE study) was registered at clinicaltrials.gov as NCT01127698. *Am J Clin Nutr* 2017;106:821–30.

Keywords: breastfeeding, clostridia, necrotizing enterocolitis, preterm infant, speed of increasing enteral nutrition

INTRODUCTION

Necrotizing enterocolitis (NEC) is one of the most dreaded diseases in neonatal intensive care units (NICUs) (1, 2). The

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Supplemental Figures 1–4, Supplemental Methods, and Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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Abbreviations used: EPIFLORE, Etude Epidémiologique de la flore; EPIPAGE 2, Etude Epidémiologique sur les Petits Ages Gestationnels; NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit; rRNA, ribosomal RNA.

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pathophysiology of NEC remains enigmatic (3). Its etiology is clearly multifactorial, with contributions from genetic predisposition, intestinal immaturity, hemodynamic instability, and intestinal microbial ecology (1–3).

A wide range of incidence of NEC from 2% to 20% has been reported (4–6). Evidence supports that feeding formula rather than human milk increases the risk of developing NEC (7). Other differences in enteral feeding, such as the timing of the introduction of feedings, the size of the daily volume of the increments, and the type of nutrients may also contribute to inter-NICU variation in the incidence of NEC. Moreover, implementation of the quality-improvement initiative reduced the NEC rate (8).

Several studies suggested that NEC is associated with both unusual intestinal microbial species and an overall reduction in the diversity of the microbiota (9–14). Fecal microbiota diversity in preterm infants born at a gestational age of <32 wk, who are at the highest risk of NEC, increases much more slowly than in more mature infants, and composition is dominated by staphylococci, enterobacteria, and enterococci, with a very low abundance of anaerobes except clostridia (15). To date, there is no consensus as to which specific bacterial strains are causally associated with NEC development. Several studies reported a dysbiosis with the phylum Proteobacteria highly represented before NEC onset (10, 11, 16). In addition, the presence of clostridia, in particular *Clostridium perfringens*, *C. butyricum*, and *C. neonatale,* has been reported either by culture (17–19) or by culture independent methods (19–23).

EPIPAGE 2 (Etude Epidémiologique sur les Petits Ages Gestationnels), a nation-wide population-based prospective cohort study (24), provided a unique opportunity to assess the role of nutritional strategies and microbiota as risk factors for NEC. We hypothesized *1*) that nutritional strategies are associated with risk of NEC and *2*) based on our previous clinical (21) and experimental (25) observations that clostridia colonization would differ between NEC cases and controls.

METHODS

Cohort EPIPAGE2 and ancillary EPIFLORE studies

EPIPAGE 2 is an ongoing birth cohort study performed in 68 NICUs in France (24). Eligible children were those born at 24–31 wk of gestation, admitted to the NICUs recruiting >10 infants (to be able to characterize the nutritional strategies of the NICU), and alive at day 7, including transferred infants. EPIFLORE (Etude Epidemiliogique de la flore) (NCT01127698) is an ancillary study of EPIPAGE 2 consisting of the establishment of a collection of stools carried out in a subset of 20 NICUs.

Ethics

This study was approved by the National Data Protection Authority (Commission Nationale de l'Informatique et des Libertés, n°911009) and by the appropriate ethics committees: the Consultative Committee on the Treatment of Information on Personal Health Data for Research Purposes (approval was granted 18 November 2010, reference number 10.626) and the Committee for the Protection of People Participating in Biomedical Research (approval was granted 18 March 2011, reference CPP SC-2873). Parents of participants provided oral, informed consent.

Outcome

The primary outcome was NEC after postnatal day 7. All infants were prospectively monitored for signs and symptoms of NEC. NEC was defined as the presence of clinical evidence fulfilling modified Bell's stage 2 or 3 criteria for NEC (26) and was confirmed by the clinical team caring for the infant. Infants who met criteria for spontaneous intestinal perforation, which differs from NEC in terms of risk factors and pathophysiology (27, 28), were excluded from this study.

Perinatal and early neonatal individual risk factors

Recorded variables included gestational age, birth weight *z* score, multiple pregnancy, gestational age, sex, birth weight *z* score based on Olsen's curves according to gestational age (29), antenatal corticosteroids, main pregnancy complications, birth in the same hospital in which NICU is located or not, mode of delivery, Apgar score, tracheal intubation at birth, respiratory distress syndrome, mode of sedation or analgesia, hemodynamic failure suggested by clinical signs and/or abnormal laboratory results and/or echocardiographic findings (30), early neonatal infections, and intestinal transit during the first week. Intestinal transit was considered regular if we observed ≥1 stool each day after the first stool during the first week (31).

Enteral feeding strategies of NICUs

We first calculated a mean expected enteral volume at day 7 with a 95% CI for each infant, according to gestational age, birth weight *z* score, and regularity of intestinal transit during the first 7 d. A low volume of enteral feeding was defined as a volume less than the lower limit of the 95% CI of the expected enteral volume at day 7. Then we calculated for each infant a probability to receive a low volume of enteral feeding at day 7 according to the characteristics of the neonate. The expected percentage of infants with a low enteral volume for each NICU was estimated by the average individual probability of each infant hospitalized in the NICU (see supplemental material). We calculated for each NICU the difference between the observed percentage and expected percentage of infants receiving a low enteral volume. We then calculated the mean ± SD of these differences for all the NICUs. A NICU with a difference between the observed and the expected percentage greater than the mean plus 1 SD of the mean was classified as slower, a NICU with a difference between the observed and the expected percentage smaller than the mean minus 1 SD of the mean was classified as faster. Other NICUs were considered as having an intermediate strategy (**Supplemental Figure 1**A).

Direct-breastfeeding strategies of NICUs

We calculated for each infant a probability to be partially direct-breastfed at day 28 according to gestational age, birth weight *z* score, and characteristics of the mother and the pregnancy. Accordingly, the expected the percentage of partially direct-breastfed infants at day 28 for each NICU was estimated by the average individual probability of each infant hospitalized in the NICU (**Supplemental Methods**). We calculated for each NICU the difference between the observed and the expected percentage. We then calculated the mean ± SD of these differences for all the NICUs. A NICU with a difference between

the observed percentage and the expected percentage higher than this mean plus 1 SD of this mean was classified as more favorable to direct-breastfeeding strategy, and a NICU with a difference between the observed percentage and the expected percentage lower than this mean minus 1 SD of this mean was classified as less favorable to direct-breastfeeding strategy. Other NICUs were considered as having an intermediate strategy (Supplemental Figure 1B).

Microbiological analysis of fecal samples in the EPIFLORE study

For infants hospitalized in NICUs participating in the EPIFLORE study, fecal samples were collected at days 7 and 28 of life. They were also collected at the time of hospital discharge in the control group. For NEC cases, a stool sample was collected at the time of diagnosis (the first stool issued after the diagnosis). Each case of NEC was matched with 6 controls for postnatal age and propensity score (see statistical analysis). Stools were collected from diapers and placed into 2 sterile tubes; the tubes for culture contained 0.5 mL brain-heart infusion broth with 15% glycerol as a cryoprotective agent. Both tubes were immediately frozen at −80°C until microbiota analysis by culture and 16S ribosomal RNA (rRNA) gene sequencing. For the culture, qualitative and quantitative analysis of fecal flora allowing the isolation, quantification, and identification of the main genera was performed by spreading dilutions of the stools on various media as described previously (32) (see Supplemental Methods). For the culture-independent method, analysis was performed by 454-pyrosequencing the V3-V4 region of the 16S rRNA gene (Supplemental Methods). DNA was extracted according to International Human Microbiome Standards SOP07 (http://www.microbiomestandards.org/fileadmin/SOPs/IHMS_SOP_07_V2.pdf).

Statistical analysis

First, we compared the individual characteristics of preterm infants with and without NEC and constructed a propensity score (Supplemental Methods) regarding the risk of developing NEC after day 7. A supplementary propensity score, including in the model enteral nutrition and perinatal antibiotics, was developed. Second, we classified NICUs according to enteral feeding advancement strategies as described above. Third, to validate the classification of NICU according to their nutritional strategies concerning speed of progression of enteral feeding, we compared the age at initiation of enteral feeding, the volume of enteral feeding at day 3, and the duration of parenteral nutrition between the 3 NICU profiles regarding the speed of progression of enteral feeding. Similarly, we compared the proportion of infants in contact with the breast of the mother during the first week and the proportion of directly breastfed infants at discharge between the 3 NICU profiles concerning direct-breastfeeding policy. Finally, to measure the association between NEC and the profiles of NICUs concerning the speed of the progression and breastfeeding policy, we used a multilevel approach to account for clustering by NICU. We performed 4 general linear mixed models for each strategy, with NEC as the outcome and the nutritional strategy of NICUs as predictors: model 1, without adjustment; model 2, with adjustment for gestational age and birth weight z score; model 3, with adjustment for gestational age, birth weight z score, and the propensity score; and model 4, with adjustment for gestational age, birth weight z score, propensity score, and the other nutritional strategy. An ancillary propensity-matched case-control study included infants hospitalized in 20 NICUs participating in the EPIFLORE study. Each NEC case was matched for the propensity score with 2 controls hospitalized in the same NICU, and 2 controls hospitalized in another NICU. We performed a second propensity score by adding 3 predictors, i.e., mode of enteral feeding at day 7 and maternal and neonatal antibiotic treatment, in the logistic regression. We matched cases based on this second propensity score with 2 other controls. Thus, in short, for each case we matched 6 controls.

RESULTS

Study population

Among the 3654 very-preterm infants admitted to NICUs during the study period, 167 died within the first 7 d, leaving 3487 alive at day 7. Among those, 3161 infants were enrolled in this study (**Figure 1**). These infants were hospitalized in 64 NICUs, 106 with NEC and 3055 controls. The median postnatal age at onset of NEC was 26 d (IQR: 20–42) (**Supplemental Figure 2**).

Individual perinatal and neonatal parameters as risk factors for NEC

Differences between the case and control infants are presented in **Table 1**. Significant risk factors associated with NEC, after adjustment for all individual variables included in the Table 1, were hemodynamic failure requiring volume expansion, an irregular intestinal transit during the first week of life, and anesthesia by sevoflurane during catheter insertion. Analgesia by morphine or surfentanil during critical care was associated with a reduced risk of NEC compared with a lack of analgesia. At the end of the first week, the propensity score significantly predicted the onset of NEC after day 7: area under the receiver operator characteristic curve = 0.79 (95% CI: 0.74, 0.84; $P < 0.001$). In this cohort, we did not observe any significant relation between maternal or initial neonatal postnatal antibiotic treatment and the onset of NEC.

Strategies of NICUs concerning speed of progression of enteral feeding and breastfeeding

The volume of enteral feeding received at day 7 was significantly dependent on gestational age ($P = 0.001$), birth weight z score ($P = 0.001$), and regular intestinal transit during the first week ($P = 0.001$). Overall, the mean difference between the observed and the expected percentage of infants per NICU receiving a low enteral volume was 2% ± 22%. Among the 64 NICUs, the difference between the observed and the expected enteral volume exceeded 24% (range: 25–48%) in 15 NICUs; these NICUs were classified as NICUs with a slower feeding strategy. In 11 NICUs the difference was <−19% (range: −41% to −20%); these NICUs were classified as NICUs with a faster strategy. The remaining 38 NICUs were classified as intermediate. The NICU patient volume was not significantly different between these 3 groups of NICUs (**Supplemental Figure 3**). Infants hospitalized in the 11 NICUs with a faster strategy received more enteral volume at day 3 and day 7. Enteral feeding started earlier, and parenteral feeding was discontinued earlier in these NICUs (**Supplemental Table 1**, **Figure 2**A).

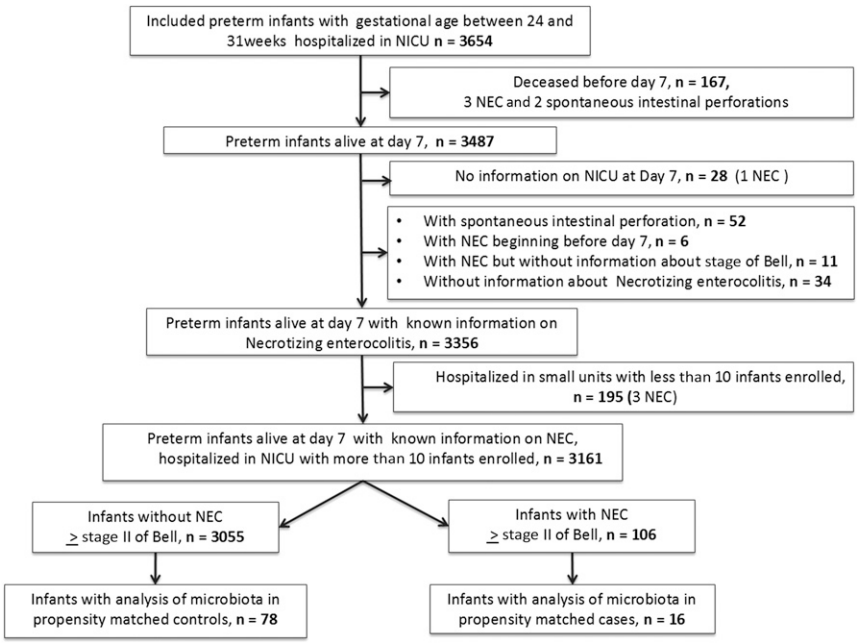

FIGURE 1 Flowchart of infants enrolled in the study. NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit.

Partial direct-breastfeeding at day 28 was significantly determined by characteristics of the mother, such as birth nationality ($P = 0.04$), professional activity ($P = 0.005$), and marital status ($P = 0.03$). It was also associated with some characteristics of the pregnancy, such as multifetal gestation pregnancy ($P = 0.007$) and hypertension during pregnancy (0.04). Partial direct-breastfeeding at day 28 was significantly dependent on characteristics of the preterm infant, such as gestational age ($P = 0.001$) and birth weight z score ($P = 0.001$). The mean difference between the observed and the expected percentage of breastfed infants at day 28 per NICU was 1% ± 8%. Among the 64 NICUs, the observed percentage of breastfed infants at day 28 was ≥−7% (range: −17% to −8%) below the expected percentage in 12 NICUs; these NICUs were classified as NICUs less favorable to breastfeeding. In 10 NICUs, the observed percentage of breastfed infants was ≥9% (range: 11–39%) above the expected percentage; such NICUs were classified as NICUs more favorable to breastfeeding. The remaining 42 NICUs were classified as intermediate. The NICU patient volume did not significantly differ between these 3 groups of NICUs (Supplemental Figure 3). Infants hospitalized in the 12 NICUs with a more favorable strategy had a higher percentage of breast contact during the first week and a higher percentage of direct-breastfeeding at discharge (Figure 2B, **Supplemental Table 2**).

Strategies of NICUs as risk factor for NEC

Slower and intermediate rates of progression for enteral feeding strategies were associated with a higher risk of NEC compared with the faster strategy after adjustment for individual risk factors and direct-breastfeeding policies ($P < 0.004$ and $P < 0.014$, respectively). The less favorable and intermediate direct-breastfeeding policies were associated with a higher risk of NEC compared with the more favorable direct-breastfeeding policy after adjustment for individual risk factors and progression of enteral feeding strategies ($P = 0.036$ and $P = 0.038$, respectively) (**Table 2**).

Microbiota and NEC

In the EPIFLORE study, 24 NEC cases were reported. Each NEC case was matched with 6 control infants for propensity score and postnatal age, ensuring that individual risk factors were not significantly different between cases and controls. Because of a lack of stools during the first 10 d after the diagnosis of NEC in 8 cases, only 16 NEC infants were sampled and matched with 78 controls. Because of inadequate fecal sampling or a too-low biomass, only 14 cases and 73 controls were analyzed by culture (**Table 3**) and 15 cases and 57 controls by the 16S rRNA gene sequencing method. The median timing of the sample collection after NEC onset was 3 d (IQR: 0–7 d, minimum: −1 d, maximum: 10 d). The median age of infants at sampling time was 24 d (range: 7–72 d) for NEC cases and 23 d (range: 7–83 d) for the controls. The median difference of postnatal age at the time of sampling between controls and cases was −1 d (IQR: −3 to 2 d).

By culture methods, at the genus level, *Clostridium* was significantly associated with NEC: 86% (95% CI: 60%, 96%) compared with 35% (95% CI: 25%, 46%) and OR: 11.3 (95% CI: 2.4, 54.4) (**Figure 3**A). At the species level, *C. neonatale* and *Staphylococcus aureus* were significantly associated with NEC: 50% (95% CI: 26%, 73%) compared with 11% (95% CI: 6%, 25%) and OR: 5.5 (95% CI: 1.4, 20.1) and 57% (95% CI: 33%, 79%) compared with 13% (95% CI: 7%, 22%) and OR: 7.1 (95% CI: 2.0, 25.0), respectively (Figure 3B).

By nonculture methods, 16S rRNA gene sequencing showed that Firmicutes and Proteobacteria were the main phyla detected with a very low contribution of Bacteroidetes and Actinobacteria in both groups. Dominant bacterial families included *Enterobacteriaceae*, *Staphylococcacae*, *Enterococcacae*, and

TABLE 1
Individual risk factors for necrotizing enterocolitis

		Cases				
Individual characteristic	N (%)[1]	n (%)[2]	OR[3] (95% CI)	P	Adjusted OR[4] (95% CI)	P
Gestational age				0.003		0.320
30–31 wk	1305 (41.3)	27 (2.1)	1		1	
27–29 wk	1224 (38.7)	49 (4.0)	1.97 (1.23, 3.17)	0.005	1.25 (0.74, 2.10)	0.404
24–26 wk	632 (20.0)	30 (4.7)	2.36 (1.23, 3.17)	0.001	0.84 (0.44, 1.62)	0.604
Birth weight,[5] g	1175 ± 348[6]					
Birth weight z score (by z score)	−0.16 (1.03)		0.79 (0.65, 0.95)	0.011	0.85 (0.70, 1.04)	0.111
Female	1507 (47.7)	43 (2.9)	0.74 (0.50, 1.10)	0.137	0.80 (0.53, 1.20)	0.278
Multiple pregnancy	985 (31.2)	38 (3.7)	1.17 (0.78, 1.76)	0.436	1.15 (0.75, 1.77)	0.515
Apgar score				0.016		0.087
>6 at 5 min	2455 (77.7)	73 (3.0)	1		1	
not known at 5 min	168 (5.3)	4 (2.4)	0.80 (0.29, 2.20)	0.660	0.66 (0.23, 1.88)	0.434
<7 at 5 min	538 (17.0)	29 (5.4)	1.86 (1.20, 2.89)	0.006	1.61 (0.99, 2.62)	0.053
Respiratory distress syndrome	1875 (59.3)	81 (4.3)	2.28 (1.45, 3.59)	0.001	1.70 (1.02, 2.84)	0.041
Hemodynamic treatment during the first week				0.001		0.001
No hemodynamic treatment	2708 (85.7)	59 (2.2)	1		1	
Volume expansion	64 (2.0)	7 (10.9)	5.51 (2.41, 12.60)	0.001	5.73 (2.42, 13.55)	0.001
Volume expansion and corticosteroids	55 (1.7)	5 (9.1)	4.49 (1.73, 11.67)	0.002	4,34 (1.56, 12.0)	0.005
Volume expansion and catecholamines	118 (3.7)	23 (19.5)	10.87 (6.44, 18.3)	0.001	11.1 (6.14, 20.11)	0.001
Catecholamines	129 (4.1)	4 (3.1)	3.74 (0.48, 29.25)	0.208	1.18 (0.40, 3.46)	0.762
Corticosteroids	13 (0.4)	1 (7.7)	1.44 (0.51, 4.02)	0.490	2.94 (0.34, 25.3)	0.325
Corticosteroids and catecholamines	53 (1.7)	7 (13.2)	6.83 (2.96, 15.76)	0.001	5.9 (2.3, 15.0)	0.001
Not known	21 (0.7)	0 (0)	—		—	
Sedation/analgesia during the first week				0.001		0.001
No sedation/analgesia treatment	2107 (66.7)	65 (3.1)	1		1	
Morphine	199 (6.3)	7 (3.5)	1.15 (0.52, 2.53)	0.737	0.37 (0.16, 0.90)	0.028
Fentanyl	146 (4.6)	6 (4.1)	1.34 (0.57, 3.16)	0.495	0.98 (0.39, 2.45)	0.961
Surfentanil	562 (17.8)	20 (3.6)	1.16 (0.70, 1.93)	0.570	0.52 (0.30, 0.92)	0.024
Ketamine	35 (1.1)	2 (5.7)	1.90 (0.45, 8.10)	0.384	1.47 (0.31, 6.91)	0.627
Midazolam	89 (2.8)	2 (2.2)	0.72 (0.17, 3.00)	0.654	0.52 (0.12, 2.24)	0.382
Sevoflurane	7 (0.2)	4 (57.1)	41.9 (9.19, 191)	0.001	29.32 (5.02, 171)	0.001
Not known	16 (0.5)	0 (0)	—		—	
Intestinal transit during the first week				0.001		0.001
Regular transit	1933 (61.2)	44 (2.3)	1		1	
Not known	197 (6.2)	1 (0.5)	0.2 (0.03, 1.6)	0.134	0.22 (0.03, 1.61)	0.135
Irregular transit	1031 (32.6)	61 (5.9)	2.7 (1.8, 4.0)	0.001	2.08 (1.34, 3.23)	0.001

[1] Values are N (%) unless otherwise indicated. N is the number of preterm infants with the characteristic; % = N/3161 infants.
[2] n is the number of preterm infants with necrotizing enterocolitis; % = n/N.
[3] Crude OR assessed by logistic regression.
[4] OR adjusted for all individual variables included in the table, assessed by logistic regression.
[5] Birth weight is not included in logistic regression.
[6] Mean ± SD.

Clostridiaceae. By contrast, *Bacteroidaceae* and *Bifidobacteriaceae* were less represented. Colonization with bacteria from the *Clostridium sensu stricto* genus tended to be associated with NEC, with an average of 20.6% of total bacteria in NEC cases compared with 11.7% in healthy controls (P = 0.08), with a higher proportion of *C. neonatale* together with *C. butyricum* (**Supplemental Figure 4**A). Gammaproteobacteria were also differentially represented with a trend toward lower proportions of *Klebsiella* and *Citrobacter* and an association with some specific bacterial operational taxonomic units related to either clostridia or Gammaproteobacteria in NEC infants (Supplemental Figure 4B).

DISCUSSION

In this nation-wide, prospective, population-based study, we identified several independent risk factors for NEC. At the NICU level, factors such as speed of the progression of enteral feeding and direct breastfeeding policy were identified. Moreover, for a given level of individual risk assessed by propensity score, differences in microbiota patterns were observed in NEC cases. A more frequent colonization by *C. neonatale* and *S. aureus* was observed by culture. A trend toward increased colonization by specific bacterial species relevant to NEC belonging to *Clostridium sensu stricto* and Gammaproteobacteria was observed by 16S rRNA gene sequencing.

Altered hemodynamics and ischemia have long been thought to play a major role and have been emphasized in some reviews (3, 33). Yet other, more recent reviews have attempted to refute such role (34). The current data seem to lend support to the "old concept" of a role of ischemia in NEC, as indicated by the significant association between circulatory failure requiring vascular repletion during the first week and the occurrence of NEC, after adjustment. Moreover, we observed a significant

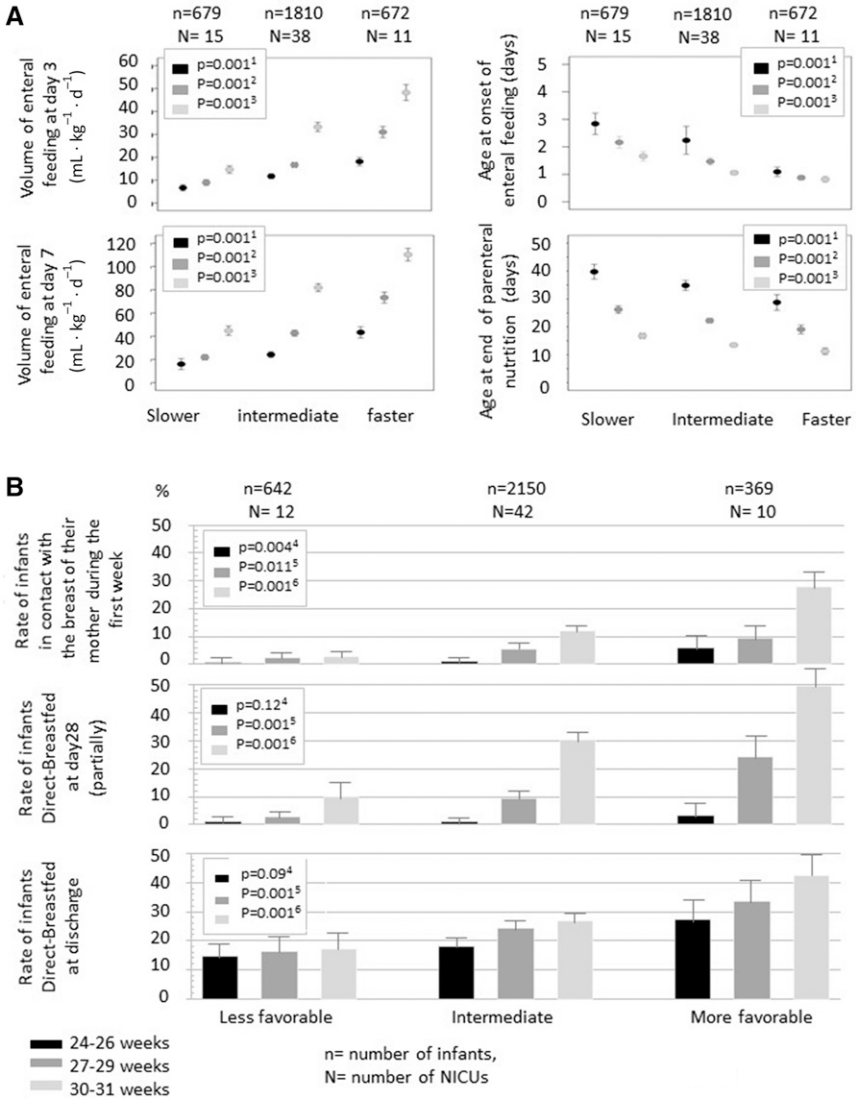

FIGURE 2 Nutritional NICU profiles concerning the rate of progression of enteral feeding policy (A). Each NICU was classified as slower, intermediate, or faster according to the difference between the observed and the expected rate of infants receiving a low enteral volume at day 7. The volume of enteral feeding at day 3 and day 7 and the age at onset of enteral feeding and at the end of parenteral feeding were compared between NICU profiles according to gestational age. Nutritional NICU profiles concerning direct-breastfeeding policy (B). Each NICU was classified as less favorable, intermediate, or more favorable to direct-breastfeeding according to the difference between the observed and the expected rate of partially direct-breastfed infants at day 28. The rate of infants in contact with the mother's breast during the first week and the rate of breastfed infants at day 28 (partial direct-breastfeeding) and at discharge (total direct-breastfeeding) were compared between NICU profiles according to gestational age. [1]*P* assessed by ANOVA for comparison among infants of 24–26 wk of gestation. [2]*P* assessed by ANOVA for comparison among infants of 27–29 wk of gestation. [3]*P* assessed by ANOVA for comparison among infants of 30–31 wk of gestation. [4]*P* assessed by chi-square test for comparison among infants of 24–26 wk of gestation. [5]*P* assessed by chi-square test for comparison among infants of 27–29 wk of gestation. [6]*P* assessed by chi-square test for comparison among infants of 30–31 wk of gestation. NICU, neonatal intensive care units.

association between anesthesia by sevoflurane and the occurrence of NEC. Hypotension and redistribution of blood flow have been observed during anesthesia by sevoflurane (35), so the mechanism likely involves a reduction in intestinal blood flow.

Differences in NICU strategies are associated with different NEC rates. Concerning the speed of the progression of enteral feeding, the evidence available from randomized controlled trials suggests that delaying the introduction of enteral feeding beyond 4 d after birth does not reduce the risk of developing NEC (36). Moreover, advancing enteral feeding volumes at daily increments of 30–35 mL/kg does not increase the risk of NEC in very-preterm or very-low-birth-weight infants (37). Advancing the volume of enteral feeding at a slow pace resulted in several days' delay in regaining birth weight and establishing full enteral feeding. Our study further suggests that the slower strategy may be deleterious. For instance, in our study in an NICU with a slower strategy, a preterm infant with a gestational age of 26 wk would only begin to receive enteral feeding at day 3 and would receive <20 mL milk/kg at 7 d of life. This strategy is associated with a higher risk of NEC. This is concordant with clinical and experimental studies, showing that a delay in enteral feeding is associated with intestinal inflammation (38) and that a small volume of feeding has little or no impact on gut growth and maturation (39). Regarding direct-breastfeeding, mother's milk has a protective effect against NEC (40). Accordingly, our study shows that the NICUs implementing a proactive strategy,

TABLE 2
Nutrition NICU policies as risk factors for necrotizing enterocolitis[1]

Nutrition NICU policies	*N*	Cases, *n* (%)	OR[2] (95% CI)	aOR[3] (95% CI)	aOR[4] (95% CI)	aOR[5] (95% CI)
Policy concerning speed of the progression of enteral feeding						
Faster speed	672	13 (1.9)	1	1	1	1
Intermediate speed	1810	62 (3.4)	1.8 (1.01, 3.2)[6]	1.8 (1.02, 3.2)[6]	1.8 (1.02, 3.2)[6]	2.0 (1.1, 3.5)[7]
Slower speed	679	31 (4.6)	1.9 (1.01, 3.6)[6]	1.9 (1.02, 3.6)[6]	1.9 (1.01, 3.6)[6]	2.3 (1.2, 4.5)[8]
Direct-breastfeeding policy						
More favorable	369	7 (1.9)	1	1	1	1
Intermediate	2150	78 (3.6)	2.0 (0.9, 4.1)	2.0 (0.9, 4.1)	2.0 (0.9, 4.1)	2.3 (1.1, 4.8)[7]
Less favorable	642	21 (3.3)	1.9 (0.8, 4.4)	1.9 (0.9, 4.4)	1.9 (0.9, 4.4)	2.5 (1.1, 5.8)[7]

[1] aOR, adjusted OR; NICU, neonatal intensive care unit.
[2] Crude OR assessed by general linear mixed models to account for clustering by NICU.
[3] OR adjusted for gestational age and birth weight *z* score, assessed by general linear mixed models.
[4] OR adjusted for gestational age, birth weight *z* score, and propensity score, assessed by general linear mixed models.
[5] OR adjusted for gestational age, birth weight *z* score, propensity score, and the other nutritional strategy, assessed by general linear mixed models.
[6] $P < 0.05$.
[7] $P < 0.03$.
[8] $P < 0.02$.

initiated from the first days of life (41), achieve a lower incidence of NEC.

The relation between microbiota and NEC has been addressed in many studies with various microorganisms implicated; however, few of them are multicenter studies. In the current study, an increased intestinal colonization by clostridia, and particularly by *C. neonatale* and *C. butyricum*, is observed at time of NEC by using both culture, despite the antibiotic therapy in cases, and 16S rRNA gene sequencing. *C. butyricum,* of which the association with NEC was first described several decades ago (42), was frequently involved. Its potential role was confirmed in a recent study combining culture and 16S rRNA gene sequencing (19). *C. neonatale*, a species closely related to *C. butyricum* (43), has been previously associated with an NEC outbreak (18). Other clostridial species can be involved. We also observed an increase in bacterial species belonging to the *Clostridium sensu stricto* genus in accordance with previous data (22). Previous studies indeed reported the overrepresentation of *C. perfringens* in NEC cases (20, 21). *C. butyricum*, *C. paraputrificum*, and *C. perfringens* were isolated in intestinal tissue specimens from neonates with NEC (17, 44), with a significant correlation between these clostridia and histologic intestinal pneumatosis in

TABLE 3
Comparison between NEC cases and controls in the ancillary propensity-matched case-control study[1]

	NEC cases (n = 14)	Controls (n = 73)	P[2]
Neonatal characteristics			
Gestational age, wk	28.4 ± 1.9	28.5 ± 1.7	0.81
Birth weight *z* score	0.05 ± 1.1	−0.38 ± 1.1	0.18
Irregular intestinal transit during the first 7 d	9 (64.3)	38 (52.1)	0.40
Apgar score <7 at 5 min	3 (21.4)	16 (21.9)	0.97
NICU profiles concerning enteral feeding			
Slower	3 (21.4)	12 (16.4)	0.78
Intermediate	9 (64.3)	45 (61.6)	
Faster	2 (14.3)	16 (21.9)	
NICU direct-breastfeeding policy			
Less favorable	2 (14.2)	12 (21.4)	0.88
Intermediate	12 (85.7)	60 (64.2)	
More favorable	0 (0)	1 (14.2)	
Microbiota			
Age at time of stool collection, d	25.8 (16.7)	23.3 (13.3)	0.55
Genus: *Clostridium*	12 (85.7)	26 (35.6)	0.001
Species			
C. neonatale	7 (50.0)	8 (11.0)	0.002
Staphylococcus aureus	8 (57.1)	10 (13.7)	0.001
C. neonatale or *S. aureus*	10 (71.4)	18 (24.7)	0.001
C. neonatale and *S. aureus*	5 (35.7)	0 (0.0)	0.001

[1] Values are means ± SDs or *n* (%). NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit.
[2] *P* values for comparison between controls and NEC cases were derived by using ANOVA, chi-square test, or Fisher's exact test as appropriate.

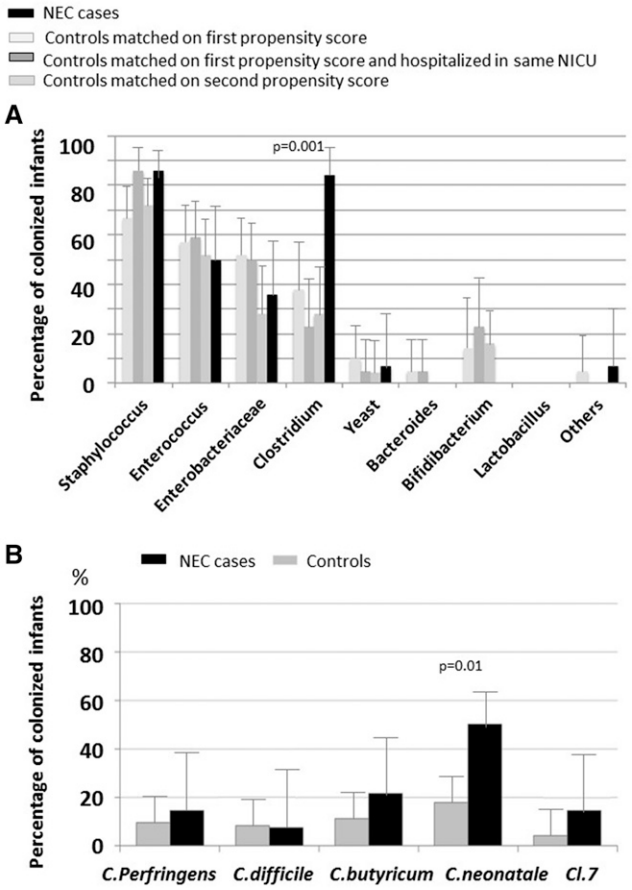

FIGURE 3 Gut microbiota associated with NEC by culture-based analyses in preterm infants enrolled in the ancillary propensity score–matched case-control study. (A) At the genus level, *Clostridium* is more often observed in NEC cases than in controls ($P < 0.001$ by chi-square test). (B) At the species level, *C. neonatale* is more often observed in cases than in controls ($P < 0.01$ by Fisher's exact test). *C., Clostridium*; *Cl.7*, other *Clostridium* species; NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit.

NEC cases (17). Very recently, the prevalence and abundance of *C. perfringens* were found increased in the meconium of neonates who subsequently developed NEC (23). Moreover, clostridial involvement in NEC onset is supported by the fact that the main clinical signs in definite NEC, i.e., intestinal necrosis and pneumatosis, are consistent with clostridial infection. Using an animal model of NEC, we previously demonstrated the role of *C. perfringens*, *C. butyricum*, and *C. paraputrificum* in the onset of the intestinal lesions (25, 45). In the current study, a strong association between clostridia and NEC was observed after adjustment for individual risk factors and NICU strategies with the use of culture and nonculture methods. The nonculture method revealed specific operational taxonomic units belonging to Gammaproteobacteria (*Enterobacteriaceae*) in higher proportions in the NEC cases. Increased levels of enterobacteria have been reported as associated with NEC without (10, 14, 46, 47) or with (20) increased levels of clostridia. This could be because of geographical distribution differences or discrepancies in NICU policies between countries. Furthermore, pathogenic characteristics leading to NEC injuries can be shared by various bacterial strains, and as suggested by 2 very recent reports, there is no specific causative taxa in NEC (48, 49).

As in all observational studies, the main limitation of our study is uncontrolled confounding bias. The nutritional strategies are not associated with the NICU patient volume, considered a proxy of NICU expertise. We cannot, however, rule out a reverse causation to explain the relation between speed of the progression of enteral feeding and NEC. Nevertheless, the current study suggests that a higher speed of progression (as used in this study) is not a risk factor for NEC and is associated with a shorter time under parenteral nutrition. Another limitation is the low number of collected stools in NEC cases. Small case series, mainly in single-center series, carry the risk of bias. However, the number of NEC cases with collected stools in the current report is of the same order of magnitude as in 14 studies included in a recent meta-analysis of NEC and microbiome (48), and the current study is a large, multicenter study involving 20 centers. The time of collection in relation to the onset of NEC constitutes another limitation, precluding any conclusion about the temporal relation between colonization by a specific bacterial strain and NEC onset. The strengths of the EPIPAGE 2 study include its population-based cohort design and prospective enrollment of all infants born prematurely in France in 2011.

In conclusion, the risk of developing NEC clearly depends on multiple individual factors. At the NICU level, the current study suggests that advancing the volume of enteral feeding at slow rates and a less favorable direct-breastfeeding policy could be deleterious. Moreover, bacterial colonization by clostridia, in particular *C. neonatale* and *C. butyricum*, is significantly more often observed in cases of NEC.

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